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Hepatitis C Virus E2-CD81 Interaction Induces Hypermutation of the Immunoglobulin Gene in B Cells†

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Hepatitis C virus (HCV) is one of the leading causes of chronic liver diseases and B-lymphocyte proliferative disorders, including mixed cryoglobulinemia and B-cell lymphoma. It has been suggested that HCV infects human cells through the interaction of its envelope glycoprotein E2 with a tetraspanin molecule CD81, the putative viral receptor. Here, we show that the engagement of B cells by purified E2 induced double-strand DNA breaks specifically in the variable region of immunoglobulin (V_H) gene locus, leading to hypermutation in the V_H genes of B cells. Other gene loci were not affected. Preincubation with the anti-CD81 monoclonal antibody blocked this effect. E2-CD81 interaction on B cells triggered the enhanced expression of activation-induced cytidine deaminase (AID) and also stimulated the production of tumor necrosis factor alpha. Knockdown of AID by the specific small interfering RNA blocked the E2-induced double-strand DNA breaks and hypermutation of the V_H gene. These findings suggest that HCV infection, through E2-CD81 interaction, may modulate host's innate or adaptive immune response by activation of AID and hypermutation of immunoglobulin gene in B cells, leading to HCV-associated B-cell lymphoproliferative diseases.

Hepatitis C virus (HCV) infection often persists despite the presence of robust host immune responses, leading to chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and B-lymphocyte proliferative disorders, including mixed cryoglobulinemia, a disorder characterized by oligoclonal proliferation of B cells, and B-cell lymphoma (44). The viral genome is a single-stranded, positive-sense RNA of 9.6 kb. The predicted structural components of the viral particles comprise the core (~21 kDa) and two heavily N-glycosylated envelope glycoproteins, E1 (~31 kDa) and E2 (~70 kDa) (17). Both E1 and E2 are type I transmembrane proteins, with N-terminal ectodomains and C-terminal hydrophobic anchors.

CD81 is thought to be a cellular receptor for HCV, based on its ability to bind E2 (21, 27, 37, 57). CD81 is a member of the tetraspanin family and is a component of the multimeric B-cell antigen receptor complex (24). It is associated with other membrane proteins, which vary in different B-cell lineages and include the signaling molecule CD19, complement receptor 2 (CD21), and interferon-inducible Leu-13 (CD225) protein (13, 24, 48). Binding of CD81 with E2 or certain monoclonal antibodies (MAbs) to CD81 induces B-cell aggregation, inhibits Daudi cell proliferation (14), stimulates T cells (45), and inhibits natural killer cell functions (7, 49). In addition, triggering of the CD81 signaling pathway in B cells enhances the production of tumor necrosis factor alpha (TNF-α) (2). Correspondingly, HCV infection of primary macrophages has been reported to induce TNF-α production (40). Coengagement of the CD19-CD21-CD81 complex and the B-cell antigen receptor lowers the B-cell activation threshold by antigen-presenting cells or lipopolysaccharide (5). Lymphocytes in mice lacking CD81 develop normally but have altered proliferative responses and are deficient in antibody production, suggesting that CD81 is one of the essential receptors for the production of antibodies (28). These observations suggest that HCV may modify the B-cell receptor-associated signaling pathway by binding to CD81.

Previously, we have reported that HCV infection induces hypermutation of many cellular genes, including immunoglobulin (Ig) and *p53* genes in B cells (25). More recent studies showed that the HCV-induced mutations of somatic genes, such as *p53*, are mediated by nitric oxide (NO), but the mechanism of HCV-induced mutation of Ig gene is not clear (26). Both the somatic hypermutation and class-switch recombination of Ig gene in normal B-cell development involve an activation-induced cytidine deaminase (AID), which triggers deamination of deoxycytidine to deoxyuracil (dU) in the template DNA strand, with preference for certain hot-spot motifs (36). The resulting dU/dG pairs can be resolved by using the mismatch repair system (35), uracil glycosylase endonuclease (8), and error-prone DNA polymerases (15). Pol ι, Pol η, and Pol ζ are involved in these pathways (11, 54, 56). Interestingly, AID, Pol ζ, and Pol ι are induced in HCV-infected B cells (25).

The exact mechanism by which HCV induces AID and other enzymes, thereby causing hypermutation of Ig gene in B cells, are not well understood. We hypothesize that the binding of HCV to CD81 may activate AID and other enzymes through the receptor-mediated signal transduction pathway. In the present study, we explored the effects of binding of E2 to CD81 on these enzymes and the accompanying changes in B cells.

MATERIALS AND METHODS

Baculovirus expression and purification of HCV E1 and E2 proteins. E1 and E2 sequences from a genotype 1a isolate (strain H77) (52) and genotype 1b

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TABLE 1. Mutation frequencies of *p53* and V_H genes in Raji cells after E2 or antibody binding[a]

Stimulation	*p53*		V_H	
	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})
Isotype	2/21	2.1*	3/26	3.1
E1	0/22	0*	4/29	3.8
E2 1a	3/29	2.3*	12/32	11.9†
E2 1b	1/18	1.2*	6/27	6.1
α-CD81	3/25	2.7*	11/35	10.1†
α-IgM + α-CD19/21	1/18	1.2*	23/32	21.3†

[a] Raji cells were incubated with E2, E1, or various antibodies on the coated plates for 5 days. V_H and *p53* gene loci from the treated cells were cloned by PCR, and multiple individual clones were sequenced as previously described (26). "Clones mutated" are the numbers of clones containing one or more mutations versus the total numbers of clones sequenced. Mutation frequencies (MF) were calculated as the total numbers of single-nucleotide mutations in all clones versus the total number of nucleotides sequenced (mutations/bp). *, The common heterozygous mutations in the *p53* gene (codon 213 CGA→CAA and codon 234 TAC→CAC) of Raji cells were excluded from the calculation. †, $P < 0.05$. The statistical significance for the excessive frequency of mutations in the cells treated with each agent versus cells treated with an isotype antibody was calculated by using the χ^2 test.

isolate (strain HC-J4) (53) without the C-terminal transmembrane domains but containing a His_6 tag at the C termini were cloned into a transfer vector (pBlueBacHis2; Invitrogen, Carlsbad, Calif.). Expression of recombinant E1 and E2 proteins in insect cells was performed as described in the Bac-N-Blue Baculovirus Expression System (Invitrogen). Insect Sf9 cells were grown in Grace's Insect medium supplemented with 10% fetal bovine serum (FBS) at 28°C. The bacmid DNAs were transfected into Sf9 cells; the virus generated was amplified two more times at a low multiplicity of infection (MOI) before being used for infection of Sf9 cells (at high MOI). At 72 h postinfection, cells were lysed in 50 mM Tris-HCl (pH 8.5), 0.15 M NaCl, 0.1% Triton X-100, and protease inhibitor cocktail (Roche, Basel, Switzerland) and then sonicated and centrifuged at 100,000 × *g*. E1 and E2 were purified from the crude lysates by using HiTrap chelating columns (Amercham Biosciences, Piscataway, N.J.) loaded with 0.1 M $NiCl_2$ (31). The extracts were diluted in 50 mM sodium phosphate (pH 8), 300 mM NaCl, and 10% glycerol (buffer A) and bound to the column (flow rate: 0.5 ml/min). The column was washed sequentially with buffer A, buffer B (50 mM sodium phosphate [pH 6], 300 mM NaCl, 10% glycerol), and buffer C (50 mM sodium phosphate [pH 8], 1 M NaCl, 10% glycerol). The proteins were eluted in buffer A containing 200 mM imidazole and buffer exchanged into phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] plus 10% glycerol.

Cells. Raji cells were obtained from ATCC. HepG2 cells were transfected with pCDNA3.1CD81 plasmid with a neomycin-resistant gene and selected with G418 (0.5 μg/ml). The surviving cell colonies were picked, and individual cell clones were characterized for CD81 expression by fluorescent-activated cell sorting (FACS). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus (Amersham Biosciences) density gradient centrifugation. PBMC were maintained in RPMI 1640 containing 10% FBS.

Western blot. Proteins were resolved by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Amersham Biosciences). The membrane was incubated with anti-His_6 (Qiagen), anti-E2 (Biodesign International, Saco, Maine), anti-AID, or anti-polymerase ι (Santa Cruz Biotechnology, Santa Cruz, Calif.) and then reacted with peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by an enhanced chemiluminescence detection system (Amersham Biosciences).

ELISA. The concentrations of the E1 and E2 (genotypes 1a and 1b) proteins in the purified preparations were assessed by enzyme-linked immunosorbent assay (ELISA) on *Galanthus nivalis* lectin (GNI; Sigma, St. Louis, Mo.) as previously described (42). For analysis of TNF-α production, 50 μl of cell culture supernatant was assayed by using an ELISA kit (Biosource International, Camarillo, Calif.) as per manufacturer's instructions.

E2 binding assays. Binding of E2 to cell surface was analyzed by a fluorescence-activated cell sorting (FACS)-based assay as previously described (14). Approximately 2×10^5 cells were washed twice in PBS–1% fetal calf serum (FACS buffer) and incubated with the partially purified E2 (10 μg) at room temperature for 1 h. After washing, a His probe (Santa Cruz Biotechnology) was added to the mixture at 2 μg/ml and incubated for 1 h at room temperature. Cell-bound His-probe was detected with anti-rabbit IgG-fluorescein isothiocyanate conjugate (Jackson Immunoresearch laboratories, West Grove, Pa.). Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.). For binding inhibition assays, cells were incubated in FACS buffer containing 20 μg of anti-CD81 MAb 1.3.3.22 (Santa Cruz Biotechnology) per ml for 30 min at room temperature, prior to incubation with E2 as described above. To determine the relative binding efficiency, a dose-response curve of E2 was determined. The percentage of cells binding E2 was derived from the best-fit analysis in the linear range of each curve.

Cell aggregation assays. Raji cells were suspended in RPMI 1640 supplemented with 20% FBS at 10^6 cells/ml and divided into aliquots into a 48-well plate (0.4 ml/well). The CD81-specific MAb, partially purified E2, or an isotype control antibody (0.1 μg/well) was added to the respective wells separately, followed by incubation at 37°C for 4 h. Cell aggregation was assessed by light microscopy and quantified by FACS determination of the FSC (forward scatter) and SSC (side scatter) values. Cell proliferation was measured by determination of viable cell counts by gating out dead cells after propidium iodide staining.

Antibody coating and cell stimulation. The cell stimulation experiments adhered to the published protocols (7). The following purified MAbs were used in the cell stimulation experiments: anti-human IgM (Caltag, Burmingame, Calif.), anti-CD19 B4 (Beckman Coulter, Fullerton, Calif.), anti-CD21 (BD Biosciences/Pharmingen) (12), anti-CD81 (JS-81; BD Biosciences), anti-E2 (Biodesign International), and anti-His_6 (Qiagen). Ninety-six-well plates were coated with the various antibodies according to published methods (7). For E2 binding, the plates were first coated with anti-E2 MAb, followed by the addition of the recombinant E2 protein and incubation at 37°C for 1 h. The excess E2 was removed by washing in PBS (−) buffer. Raji cells (5×10^4) were added to the coated plates, followed by incubation for various periods of time as indicated in the experiments. For the experiments involving the blocking anti-CD81 antibody, Raji cells were first treated with blocking antibody (clone 1.3.3.22) at 37°C for 30 min. The cells were washed once and then added to the E1, E2, or antibody-coated plates as described above.

LM-PCR analysis of DSBs. A pair of primers, BW-1 (5′-GCGGTGACCCG GGAGATCTGAATTC-3′) and BW-2 (5′-GAATTCAGATC-3′) were used as linkers and ligated to the blunt ends of DNA generated by double-strand DNA breaks (DSBs) (43). For quantitation of broken DNA ends, linker-ligated DNA was serially diluted twofold (30, 15, and 7.5 ng of linker-ligated DNA) and used for LM-PCR (55). ApoAlert ligation mediated-PCR (LM-PCR; Clontech, Palo Alto, Calif.) was used for this purpose. To detect the V_H- or *p53*-specific DSBs, the locus-specific primers for either V_H or *p53* were used (25). The blots were hybridized to the appropriate [γ-^{32}P]ATP-end-labeled probes for detecting the V_H region or *p53* gene, respectively.

Reverse transcription-PCR (RT-PCR). Raji cells were lysed with TRI reagent (Molecular Research Center, Cincinnati, Ohio). One microgram of the total RNA was reverse transcribed by using oligo(dT) primers (New England Biolabs, Beverly, Mass.) and Superscript II enzyme (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. The cDNA was diluted fivefold with water sequentially two times; 1 μl each from these dilutions was used in a PCR. *Taq* polymerase (Roche Diagnostics, Indianapolis, Ind.) was used for amplification with primers specific for *pol* ζ (54), β-*actin* (54), *pol* ι (38), and *AID* (41). HCV RNA was detected by a procedure described in the previous report (46). The PCR products were analyzed by electrophoresis on 2% agarose gel. Gels were scanned, calibrated, and quantified by using ChemiImager 4400 software (Alpha Innotech, San Leandro, Calif.). Each dilution at each time point was normalized by using β-*actin* amplified from the cDNA at that dilution.

Quantitation of *polymerase* ζ mRNA by RT-PCR. Twenty nanograms of cellular RNA was used to determine the mRNA level of the catalytic subunit *REV3* of human DNA polymerase ζ by quantitative real-time TaqMan PCR. The data analysis was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, Calif.). Sense and antisense primers, together with a reaction-specific fluorochrome-labeled probe, were synthesized as previously described (51). The *GAPDH* levels were included as a control in each reaction. In all analyses, only differences of >4-fold (>2 PCR cycles) were considered significant.

Genomic DNA cloning and sequencing. Genomic DNA from the various cell lines was extracted according to standard methods. PCR amplification was performed by using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.) and the reported primers for V_H (from V_H framework 1 to constant region 1 of heavy-chain genes) and *p53* (exons 5 to 8) (26). The purified PCR products were further incubated with *Taq* polymerase (Roche Applied Sciences, Indianapolis, Ind.) and 0.2 mM dATP for 15 min at 72°C. The PCR products were ligated into the TOPO cloning vector (Invitrogen), and individual clones containing an insert

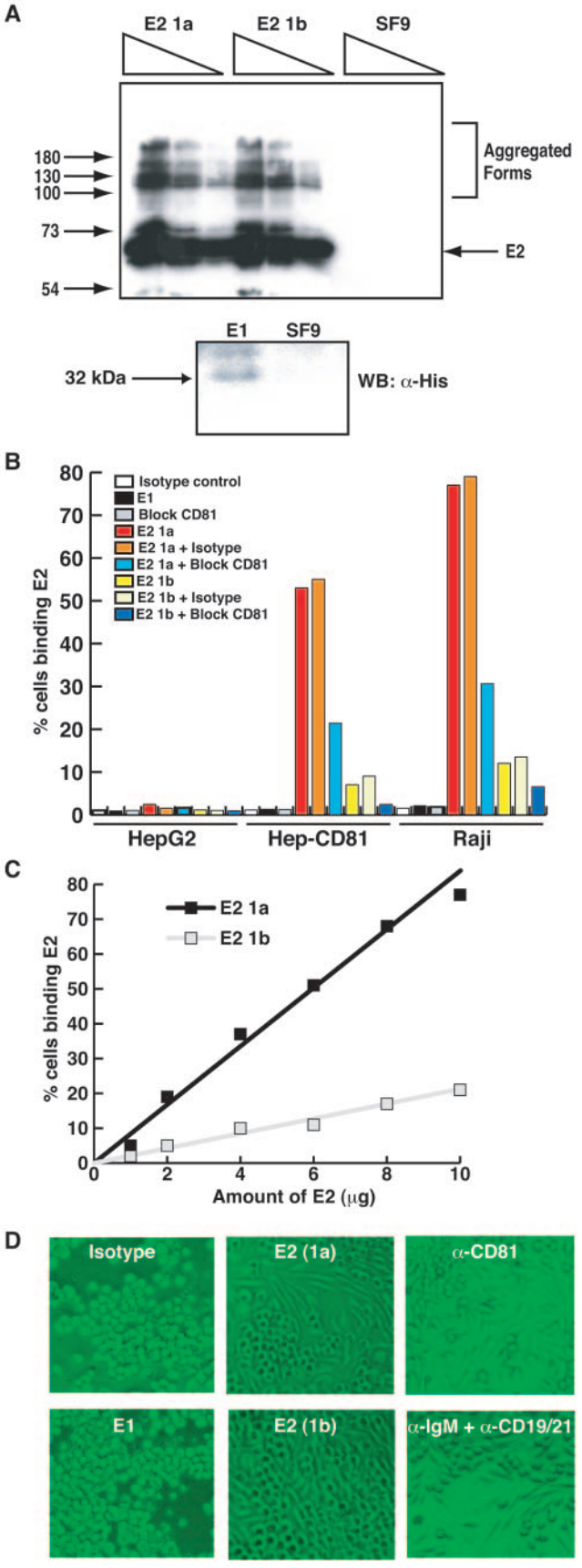

FIG. 1. CD81 interacts with recombinant HCV E2 protein. (A) Lysates from Sf9 cells expressing HCV E2 or E1 proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. E2 or E1 was detected by immunoblotting with anti-E2 MAb or His probe, respectively. Monomeric and aggregated E2 species are indicated. (B) Binding of E2 to Raji and Hep-CD81 cells in the presence or absence of various antibodies. The percentage of cells binding E2 was measured by FACS. Average values from two replicates are presented. (C) Dose-response curve for E2 binding to Raji cells. Different amounts of E2 from genotypes 1a and 1b were used in the binding experiment as in panel B. (D) Morphological changes of Raji cells at 48 h after treatment with E1, E2, or various antibodies.

of the expected size were sequenced (Laragen, Inc., Los Angeles, Calif.). GenBank sequence for *p53* is under accession number U94788.

RNA interference using siRNA. The small interfering RNAs (siRNAs) used for AID and polymerase ι (synthesized by the USC Microchemical Core, Los Angeles, Calif.) were designed according to the guidelines from Elbashir et al. (10) based on the reported sequences (25). Raji cells were transfected with the siRNAs and incubated as previously described (47). Briefly, 2×10^4 cells were suspended in 50 μl of serum- and antibiotic-free RPMI medium and then cultured in a 96-well tissue culture dish. A preincubated solution of Oligofectamine (Invitrogen) containing 100 pmol of siRNA (50 μl in total volume) was added to Raji cells, followed by incubation overnight at 37°C (47). Cells were transfected with the same siRNA again on day 3. One day after the second transfection, cells were used for E2 binding. Nonfunctional siRNA (Ambion) was used as a control. Transfection efficiency was determined by using a control (nonsilencing) siRNA labeled with rhodamine (Qiagen, Valencia, Calif.) to be >70%.

Inhibition of polymerase ζ expression by specific polymerase ζ REV3 oligonucleotides. The phosphorothioate-modified oligodeoxynucleotides specific for the polymerase ζ catalytic subunit, REV3 (54), were synthesized (Integrated DNA Technologies, Coralville, Iowa) and transfected into Raji cells as described previously. After incubation for 24 h at 37°C, Raji cells were washed and used for the E2 binding experiments. The number and viability of cells (trypan blue exclusion) were determined. More than 80% of the Raji cells were viable at any time points.

Annexin V staining. Cells were incubated in the labeling solution containing fluorescein isothiocyanate-labeled annexin V (Oncogene Research Products, San Diego, Calif.) as described by manufacturer and analyzed with FACSCalibur (Becton Dickinson).

Statistical analysis. Statistical analysis of the data in Tables 1, 2, and 3 was performed by using the χ^2 test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Recombinant E2 from genotypes 1a and 1b isolates binds to cells in a CD81-dependent manner. To characterize the E2 binding to CD81, the His-tagged E2 (without the transmembrane domain) from genotypes 1a and 1b were expressed by the baculovirus expression system and partially purified. The intracellular E2 was detected in both monomeric and aggregated forms (Fig. 1A). The partially purified E2 was used for in vitro binding to various cell lines. The percentages of cells binding E2 were determined by flow cytometry. The results showed that E2 from genotype 1a bound to Raji cells, an established human B-cell line, very efficiently. E2 from genotype 1b also bound, but less efficiently (Fig. 1B), in agreement with a previous report (42). The binding strengths of E2 of genotypes 1a and 1b were further compared by using different amounts of E2 (the amounts of E2 were normalized by using an anti-His_6 antibody). As shown in Fig. 1C, E2 of genotype 1a bound three to four times more than that of genotype 1b at

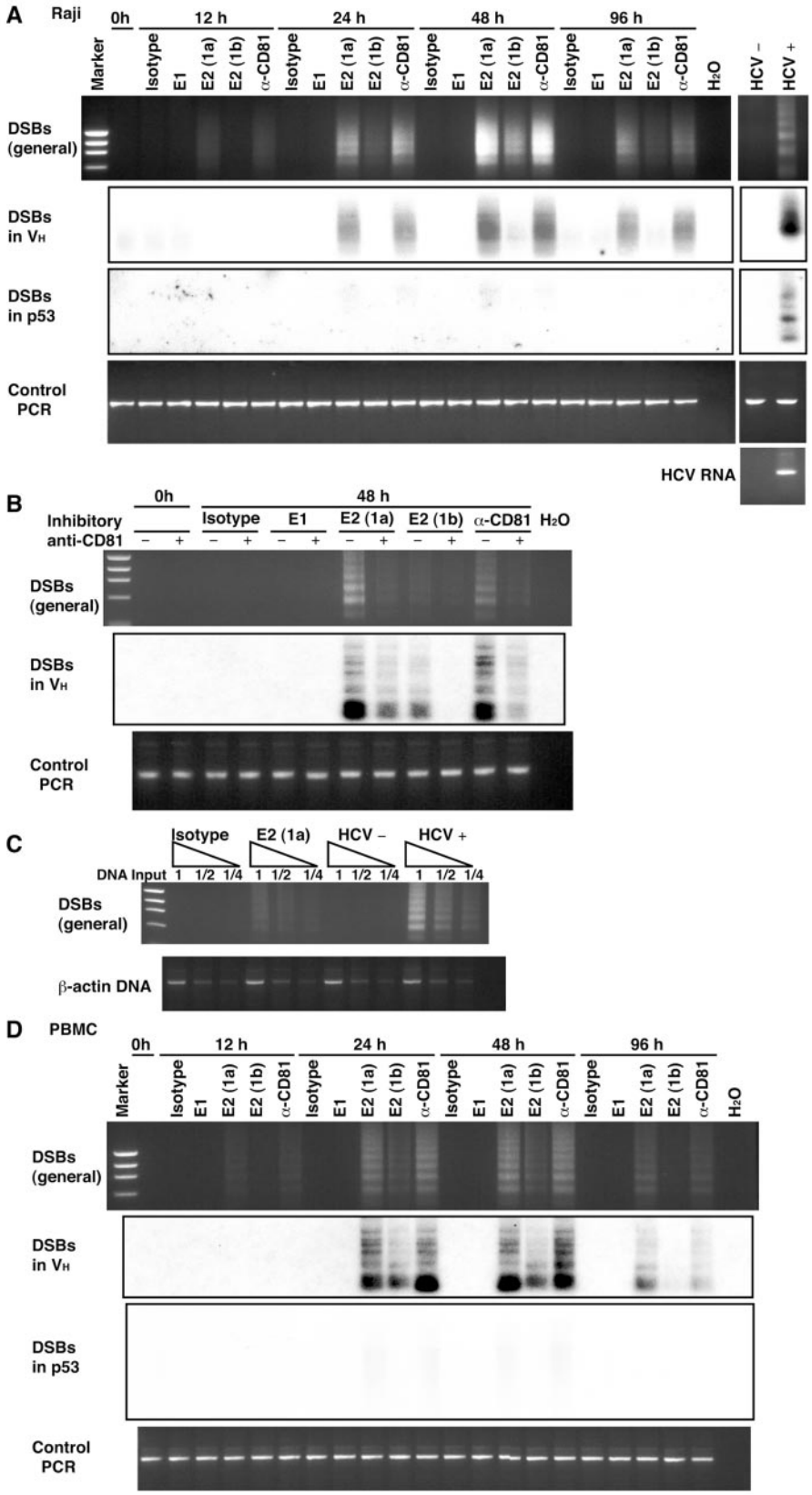

FIG. 2. E2 binding induces DSBs in Raji cells and PBMC, as determined by ligation-mediated PCR (LM-PCR). (A) Cells were treated with E1, E2 (genotypes 1a and 1b), or anti-CD81 antibody, and the cellular DNA was used for LM-PCR for detecting DSBs in general or in V_H or *p53* specifically (see Materials and Methods). DNA from HCV-infected or uninfected Raji cells was used as a control. HCV RNA was detected with RT-PCR. Control PCR with β-*actin* served as an internal control. (B) DSBs in cells pretreated with (+) or without (−) an inhibitory anti-CD81 antibody before binding with E1, E2, or a stimulatory anti-CD81 antibody. (C) Comparison of DSBs in E2 (1a)-treated or HCV-infected Raji cells. DNA samples were serially diluted. (D) DSBs in PBMC. The conditions of treatment were the same as in panel A.

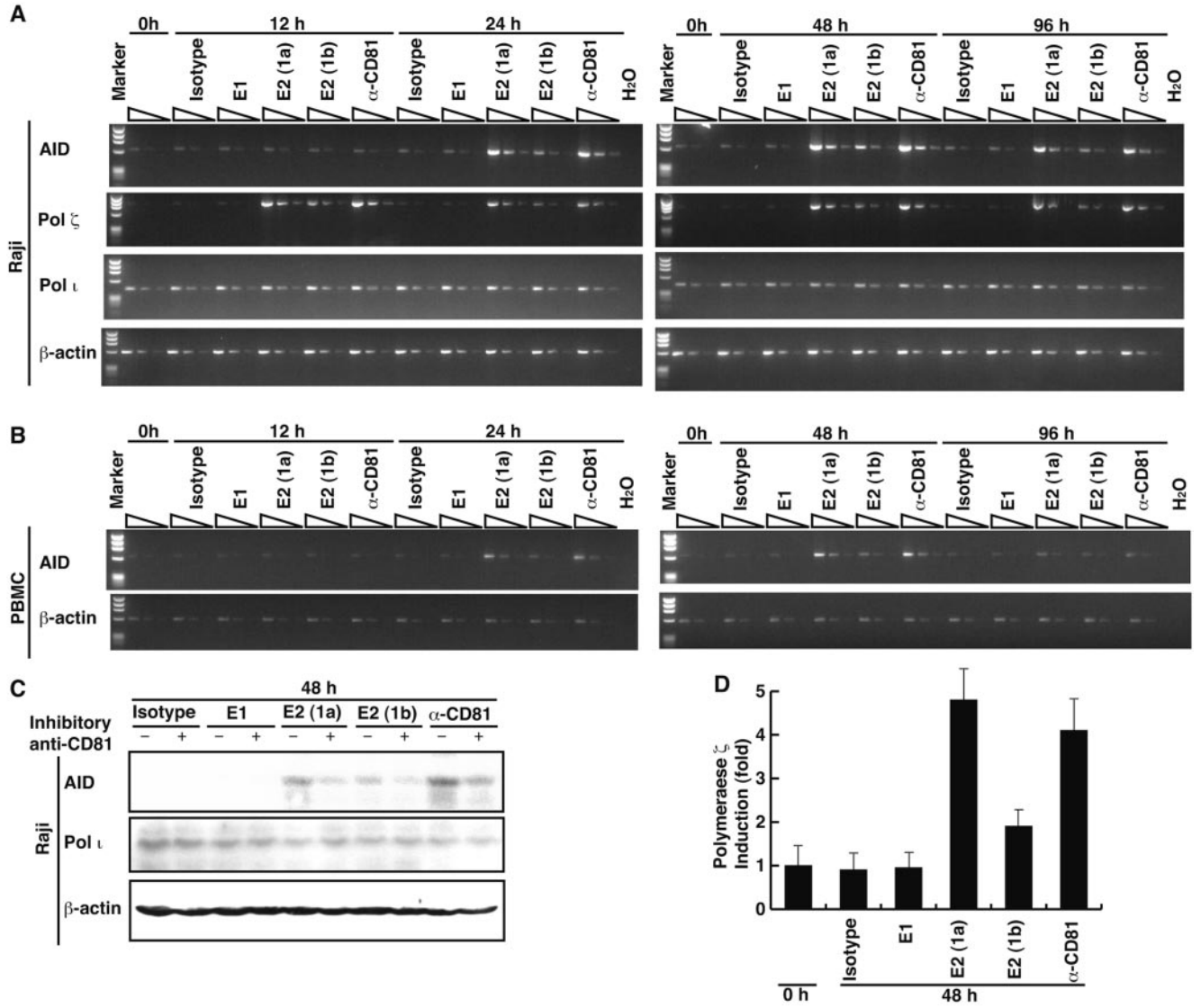

FIG. 3. Induction of *AID* and error-prone DNA polymerases in Raji cells and PBMC by E2 binding. (A) RNA samples from Raji cells at different time points after the various treatments were used for semiquantitative RT-PCR amplification of *AID*, *polymerase* ζ, *polymerase* ι, and β-*actin*. The cDNA of different dilutions (no dilution, 1:5 and 1:25) was used for PCR amplification. H_2O, water control for PCR. (B) AID expression in PBMC. (C) AID and polymerase ι protein expression in Raji cells as detected by immunoblotting. (D) Real-time quantification of *polymerase* ζ mRNA in Raji cells. The degrees of enhancement after the various treatments are shown.

every protein concentration used. E2 did not bind HepG2 cells, which are among the few human cell lines that do not express CD81. In contrast, E2 bound the CD81-overexpressing HepG2 cells efficiently. The binding was partially blocked by a CD81-specific antibody but not by an isotype control antibody (Fig. 1B). These results indicate that E2 binds to B cells in a CD81-dependent manner. We have also performed similar studies with the recombinant E1 (Fig. 1A). No binding by E1 protein to any of these cells was detected (Fig. 1B).

We next examined whether E2 binding induced morphological changes of B cells. Immobilized E2 induced the aggregation and spindle-shape formation of Raji cells, which was visible under a microscope at 48 h after binding (Fig. 1D). Cell aggregation was confirmed by changes in FSC and SSC in FACS (data not shown). As a positive control, immobilized anti-CD81 MAb or anti-CD19/21+IgM antibodies also induced the similar morphological changes in Raji cells (Fig. 1D). These results combined indicate that the binding of E2 to Raji cells is CD81 dependent and leads to cell aggregation and morphological changes, in agreement with previous reports (14, 24).

E2-CD81 interaction induces DSBs in V_H genes. We have recently reported that HCV infection induces DSBs and mutations in *p53*, β-*catenin*, *bcl-6*, and V_H genes (25). However, although the DSBs in *p53* could be largely inhibited by iNOS inhibitors, those in Ig could not be inhibited by the same treatment (26). Furthermore, overexpression of HCV proteins core and NS3 induced DSBs and mutations in the *p53* gene, but none of the viral proteins could do the same in the V_H gene (26). Therefore, the DSBs in the V_H gene are likely caused by

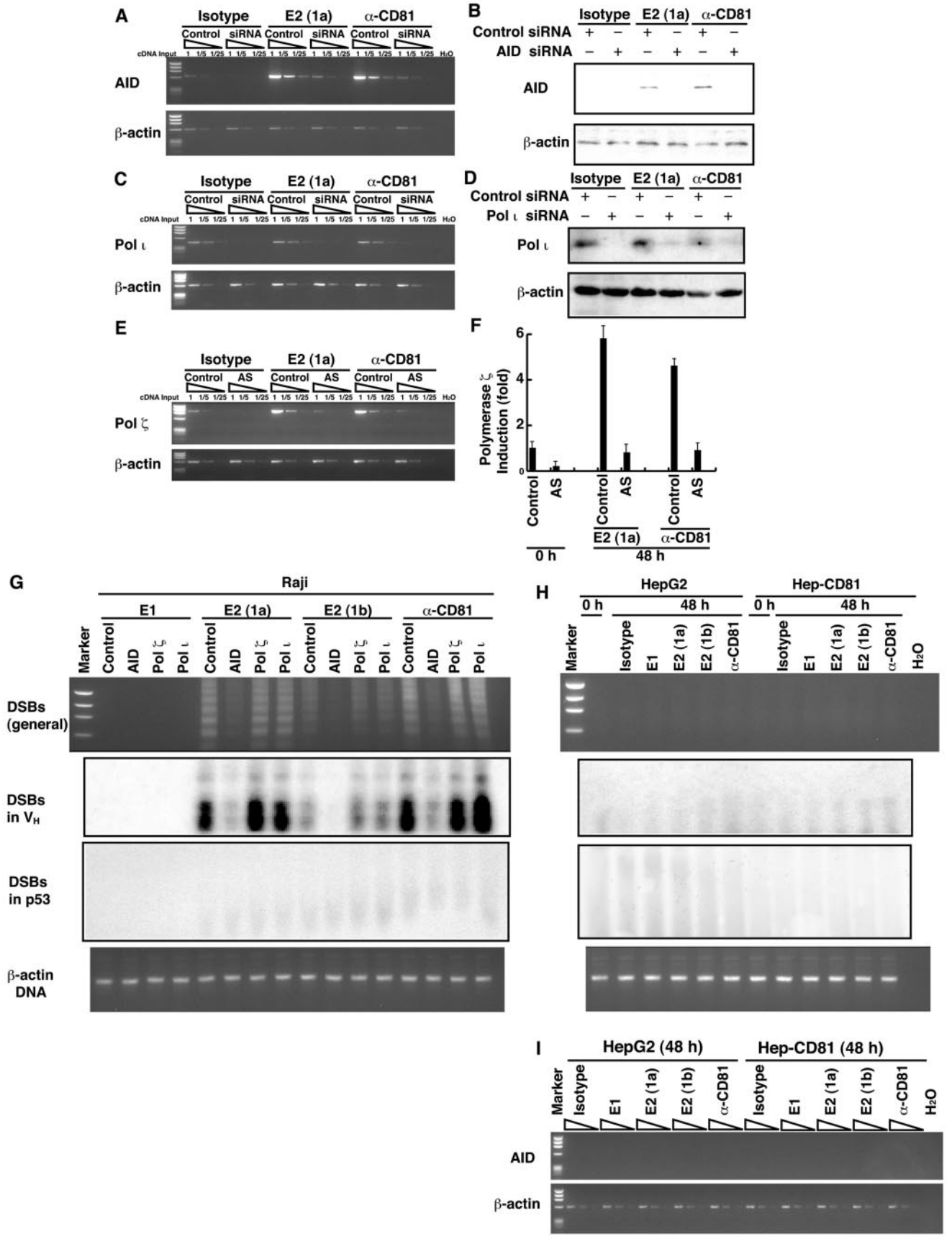
A
Isotype
E2 (1a)
α-CD81
Control siRNA
cDNA Input
AID
β-actin
B
Control siRNA
AID siRNA
C
Pol ι
D
E
AS
Pol ζ
F
Polymerase ζ Induction (fold)
0 h
48 h
G
Raji
E1
E2 (1a)
E2 (1b)
Marker
DSBs (general)
DSBs in V_H
DSBs in p53
β-actin DNA
H
HepG2
Hep-CD81
I
HepG2 (48 h)
Hep-CD81 (48 h)
H2O

TABLE 2. Mutation frequencies of V_H gene in Raji cells transfected with various siRNA/antisense oligodeoxynucleotides and followed by E2 or antibody binding[a]

Silenced target	Isotype		E1		E2 1a		E2 1b		α-CD81	
	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})
Control	2/20	2.7	2/21	2.6	11/23	14.2	5/20	6.8	10/24	11.4
AID	1/24	1.1	1/22	1.2	4/24	4.5*	2/20	2.7	3/20	4.1*
Polymerase ζ	3/21	3.9	3/20	4.1	10/24	11.4	6/25	6.5	8/21	10.4
Polymerase ι	2/23	2.4	2/24	2.3	8/23	9.5	4/24	4.6	7/21	9.1

[a] Raji cells were transfected with siRNA/antisense oligodeoxynucleotides against AID, Polymerase ζ or Polymerase ι or a control siRNA. Five days later, cells were incubated with E2, E1, or isotype or anti-CD81 antibodies as indicated. At 5 days after treatment, the cells were harvested. The mutation frequency (MF) and the number of mutations in the V_H gene were determined as described in Table 1. *, $P < 0.05$. The statistical significance for the excessive frequency of mutations in each gene of cells treated with siRNA/antisense oligodeoxynucleotides versus control siRNA was calculated by using the χ^2 test.

a mechanism different from that in the *p53* gene. We therefore considered the possibility that the E2-CD81 interaction may induce DNA damage in the V_H gene locus. We examined DSBs in Raji cells at various time points after E2 binding by using the LM-PCR method. Significantly, E2 binding of Raji cells induced DSBs, which were detectable beginning at 24 h posttreatment and reached the highest level at 48 h posttreatment. The degrees of DSBs subsequently declined. The extent of DSBs induced by E2 of genotype 1a was very similar to that induced by an agonistic CD81-specific antibody (Fig. 2A). E2 of genotype 1b induced a relatively lower level of DSBs, a finding consistent with its weaker CD81-binding capacity. E1 or the isotype-control antibody did not induce DSBs. We next examined whether the DSBs induced by E2 binding were found in both *p53* and V_H gene loci. The results showed that a high degree of DSBs were detected in the V_H region, but only a background level of DSBs was found in the *p53* gene locus (Fig. 2A). All of the DNA samples were normalized with respect to β-*actin* (Fig. 2A). Pretreatment of cells with a blocking MAb to CD81 prior to E2 binding efficiently blocked the induction of DSBs in V_H (Fig. 2B). The extent of DSBs induced by E2 binding to Raji cells was less than that observed in the HCV-infected Raji cells at 16 days after infection (25) (Fig. 2C). Finally, DSBs in the V_H locus were not due to apoptosis, since we have previously shown that the nonapoptotic cells from the HCV-infected cells also contained enhanced DSBs (25). These results combined indicate that E2-CD81 interaction causes DSBs in the genomic DNA, specifically in the V_H locus.

To rule out the possibility that the E2-induced DSBs were an artifact of cultured cell lines, we also performed similar studies on PBMC from healthy individuals. As shown in Fig. 2D, E2 of both genotypes 1a and 1b induced DSBs in PBMC to an extent similar to that induced by the agonistic anti-CD81 antibody. E2 of genotype 1b induced a lower level of DSBs, similar to the finding in Raji cells. The kinetics of DSB induction in PBMC was similar to that seen in Raji cells. Also, DSBs were seen only in the V_H gene and not in the *p53* gene locus. Therefore, the E2-induced DSBs likely occur in natural HCV infections of B cells. These findings contrasted with the previous finding that HCV core and NS3 proteins induced DSBs strongly in *p53* but weakly in V_H (26).

E2-CD81 binding induces activation-induced cytidine deaminase (*AID*) and error-prone DNA polymerase ζ. We have previously shown that HCV infection induced the expression of AID, which plays a key role in the hypermutation of Ig in B cells (36). Furthermore, the introduction of the siRNA for AID inhibited mutations of Ig (25). We therefore determined whether AID could be induced by E2-CD81 binding. Semiquantitative RT-PCR analysis showed that the binding of E2 of genotypes 1a and, to a lesser extent, genotype 1b stimulated the expression of *AID* mRNAs in Raji cells beginning at 24 h posttreatment (Fig. 3A) and peaking at 48 h. Its expression level was similar to that seen in the cells treated with the agonistic CD81-specific antibody. Similarly, E2 also induced AID mRNA expression in PBMC (Fig. 3B). The enhanced expression of AID was also confirmed by immunoblotting with AID-specific antibodies; the binding of E2 of both genotypes 1a and 1b clearly enhanced the amount of AID protein in Raji cells compared to the cells treated with E1 or the isotype antibody (Fig. 3C). We also tested whether the intracellular expression of E2 or any other HCV proteins could induce AID expression; none of these viral proteins did (Fig. S1 in the supplemental material). Finally, pretreatment with a blocking MAb against CD81 abrogated the induction of AID by E2 (Fig. 3C). These results established that AID was induced by extracellular stimulation by E2 but not by intracellular viral protein expression.

We next examined the status of error-prone DNA polymerases since error-prone polymerases ζ, η, ι, and μ have been postulated to be the mutagenic polymerases for somatic hypermutation (11, 15, 54) during repair of DSBs or single-strand DNA breaks (3, 23, 34). Previously, we have shown that HCV infection induces polymerases ζ and ι (25). Therefore, we pro-

FIG. 4. Effects of silencing of AID and polymerases ι and ζ on DSBs. (A to D) The silencing of AID and polymerase ι expression by siRNA transfection in Raji cells as determined by RT-PCR of RNA (A and C) and immunoblotting of proteins (B and D). Samples were collected 2 days after stimulation by E2. (E and F) The expression of *polymerase* ζ mRNA in Raji cells transfected with the specific antisense oligodeoxynucleotide (AS) as determined by semiquantitative RT-PCR (E) or real-time quantitative RT-PCR (F). (G) E2-induced DSBs in Raji cells after siRNA or antisense DNA transfection as in panels A to F. (H) DSB formation in HepG2 or Hep-CD81 cells treated with E1, E2 or anti-CD81 antibody. (I) Semiquantitative RT-PCR of *AID* and β-*actin* transcripts in HepG2 or Hep-CD81 cells after the various treatments.

TABLE 3. Mutation frequencies of the V_H gene in Raji cells treated with a blocking anti-CD81 antibody followed by E2 or antibody binding[a]

Antibody	Isotype		E1		E2 1a		E2 1b		α-CD81	
	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})	Clones mutated	MF (10^{-4})
Control MAb	2/22	2.5	2/23	2.4	10/19	14.4	4/23	4.8	9/20	12.3
Blocking α-CD81	2/24	2.3	3/22	3.7	5/24	5.7*	3/23	3.6	4/18	6.1

[a] Raji cells were incubated with a blocking anti-CD81 antibody (clone 1.3.3.22) or a control MAb for 30 min. After the antibodies were removed, the cells were further incubated with E1, E2, or a stimulatory anti-CD81 antibody. At 5 days after the treatment, the mutation frequency (MF) of V_H was determined as described in Table 1. *, $P < 0.05$. The statistical significance for the excessive frequency of mutations in cells pretreated with a blocking CD81 MAb versus those in the cells pretreated with a control antibody was calculated by using the χ^2 test.

ceeded to determine the expression levels of these two polymerases by semiquantitative RT-PCR of their transcripts after the E2 binding. The results showed that the expression level of *polymerase* ζ was approximately five times higher in the E2-bound cells than that in the cells treated with the isotype antibody or E1 protein by 12 h posttreatment (Fig. 3A). This finding was confirmed by quantitative real-time RT-PCR determination of the *polymerase* ζ transcript (Fig. 3D). Because of the poor quality of the antibodies against polymerase ζ, we could not determine the extent of polymerase ζ protein induction (344 kDa) by immunoblotting. In contrast, when E2 was expressed in the cells, the expression of AID or *polymerase* ζ was not elevated (Fig. S1 in the supplemental material). These data together indicated that error-prone DNA *polymerase* ζ is activated by the binding of E2 to cell surface. However, the expression level of *polymerase* ι was not elevated.

E2 binding induces hypermutation of V_H in B-cell lines. To examine the possible association between the E2-CD81 interaction and somatic mutations of cellular genes, we determined mutation frequency of the V_H and *p53* gene loci in Raji cells after incubation with E2. V_H gene was amplified by PCR, and multiple PCR clones were sequenced. Significantly more point mutations were found in the V_H gene of the cells incubated with the partially purified E2 (genotype 1a) than those in the cells treated with E1 or isotype antibody (Table 1). The mutation frequency of V_H locus in the E2-treated cell was calculated to be 11.9×10^{-4} mutations per base pair (mutations/bp), which was close to that in the cells treated with anti-CD81 antibody (10.1×10^{-4} mutations/bp) but nearly three to four times higher than those treated with an isotype-control antibody or E1 protein (3.1 to 3.8×10^{-4} mutations/bp) (Table 1). E2 of genotype 1b caused a lower degree of increase in mutation frequency. In contrast, the binding of E2 or any other molecules tested did not alter the mutation frequency of *p53* locus significantly, indicating that E2 binding induces mutations in V_H, but not *p53* gene.

siRNA targeting *AID* reduced the mutations of V_H genes. We next used the RNA interference and antisense strategy to test the hypothesis that the extracellular E2 enhanced mutations of V_H gene through activation of AID or error-prone DNA polymerases. Toward this end, we evaluated the effects of siRNAs targeting AID and polymerase ι, as well as of antisense oligodeoxynucleotides targeting polymerase ζ. The introduction of the AID siRNA significantly reduced the degree of the E2- or anti-CD81-induced enhancement of the expression of the AID mRNA (Fig. 4A). Correspondingly, the production of AID protein in the cells treated with E2 or anti-CD81 antibody was almost completely blocked (Fig. 4B). The expression of polymerase ι mRNA and protein was also inhibited by the polymerase ι-specific siRNA (Fig. 4C and D). Similarly, the introduction of the *polymerase* ζ antisense oligodeoxynucleotide resulted in at least a sixfold reduction in the expression level of polymerase ζ transcript in the cells (Fig. 4E and F). We then determined whether these siRNAs and antisense oligodeoxynucleotide could block the DSBs induced by E2 or anti-CD81 antibody. Figure 4G shows that the siRNA targeting AID blocked the occurrence of DSBs, specifically in the V_H gene, induced by E2 or anti-CD81 antibody. Surprisingly, nei-

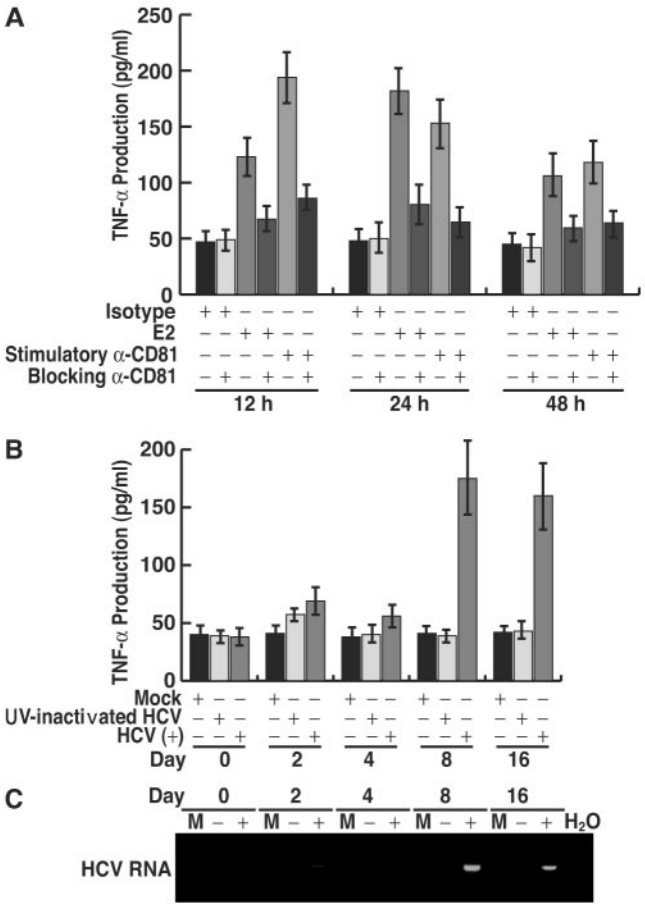

FIG. 5. E2-CD81 interaction induces TNF-α production by Raji cells. (A) TNF-α production as determined by ELISA at various time points after E2 or antibody binding. (B) TNF-α production after HCV infection. (C) Detection of HCV RNA in infected cells by RT-PCR. M, mock.

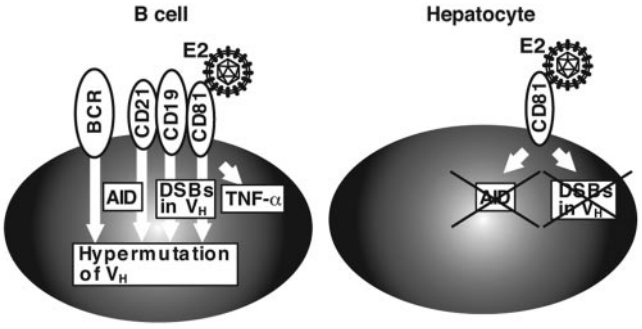

FIG. 6. A postulated signaling pathway for induction of hypermutation of Ig gene in B cells or hepatocytes by E2 binding. BCR, B-cell receptor complex.

ther the antisense oligodeoxynucleotides against polymerase ζ nor the siRNA against polymerase ι affected the DSB formation. These results indicate that AID or its downstream gene product is the enzyme responsible for the occurrence of DSB in the V_H gene, whereas polymerases ζ and ι are not involved in this process, even though the expression of polymerase ζ is enhanced by the binding of E2 or anti-CD81 antibodies.

The mutation frequency of the V_H locus in the AID siRNA-treated cells after binding with E2 (genotypes 1a and 1b) or anti-CD81 antibody was significantly lower than that in the corresponding cells treated with the control siRNA (Table 2). In contrast, neither the antisense oligodeoxynucleotide against polymerase ζ nor the siRNA against polymerase ι significantly affected the mutation frequency of V_H induced by the E2 or anti-CD81 antibody (Table 2). These results correspond very well with the finding that polymerases ζ and ι did not play a role in the E2-induced DSB formation. Thus, AID is responsible for most of the E2-induced mutations in the V_H region, whereas polymerases ζ and ι do not significantly affect the mutation frequencies, even though the expression of polymerase ζ is enhanced by E2 binding.

Pretreatment of Raji cells with a blocking CD81 MAb partially blocked the E2- or anti-CD81 (agonist) antibody-induced enhancement of mutation frequency of V_H (Table 3). These results establish that E2-CD81 interaction induces hypermutation of Ig gene.

We further sought to determine whether the E2-induced DSB was specific for B cells or a general phenomenon. We performed the E2 binding experiment in HepG2 cells and HepG2 cells expressing CD81. As shown in Fig. 4H, none of the treatments induced any detectable DSB, even though E2 and anti-CD81 antibody bound to Hep-CD81 cells very well (Fig. 1B). Also, none of these treatments induced the expression of *AID* transcript (Fig. 4I). These results indicate that the E2-induced DSBs and the subsequent V_H mutations are specific for B cells.

E2-CD81 binding stimulates the secretion of TNF-α by Raji cells. To detect other possible consequences of E2 binding to B cells, we examined TNF-α production by B cells after E2 binding, since the engagement of B cells by anti-CD81 antibodies has been shown to induce TNF-α production (2). The amount of TNF-α secreted into the culture supernatants of Raji cells treated with E2 of genotype 1a was determined by ELISA. In agreement with a previous report (2), incubation of Raji cells with a stimulatory anti-CD81 antibody induced a three- to fourfold increase in the amount of TNF-α produced (Fig. 5A). The enhancement of TNF-α production was blocked by pretreatment with a blocking anti-CD81 antibody. Binding of E2 to Raji cells also enhanced the release of TNF-α to a similar extent. TNF-α production peaked at 12 to 24 h after treatment. Treatment of cells with an anti-CD81 blocking antibody prior to E2 binding reduced the TNF-α level (Fig. 5A). These results indicate that the engagement of B cells by E2 stimulates TNF-α production. To establish the biological relevance of the E2-induced TNF-α production, we further investigated whether HCV infection of Raji cells also induced TNF-α production. The virus inoculation induced a slight increase of TNF-α production on day 2 postinfection. A more significant increase was observed on day 8 and continued until day 16 (Fig. 5B), when HCV RNA could be detected in the infected cells (Fig. 5C). In contrast, UV-inactivated virus [RNA(−)] did not induce TNF-α production (Fig. 5B). These results indicate that HCV E2 protein, through its binding to CD81, is a potential inducer of TNF-α production in natural HCV infection.

DISCUSSION

This study shows that E2-CD81 interaction induces AID and DSBs, which lead to hypermutation of V_H in B cells. Furthermore, this interaction induces TNF-α production by B cells. These effects were confirmed in the natural HCV infection of B cell. These findings implicate that, even in the absence of virus replication, the very act of virus binding to B cells can contribute to the pathogenesis of HCV. AID has been shown to promote illegitimate DNA recombination and somatic mutations of both Ig and non-Ig genes; thus, aberrant expression of AID is potentially oncogenic (32). Indeed, aberrant expression of AID has been associated with chromosomal aberrations in patients with lymphocytic leukemia and non-Hodgkin's B-cell lymphomas (18, 20), although the functional significance of this association has not been established (1). In addition, transgenic mice with ectopic and dysregulated AID expression die early because of the development of epithelial and lymphoreticular neoplasm harboring hypermutated Ig and non-Ig genes (32). AID may initiate DNA breaks, which are mediated by uracil-DNA glycosylase and apyrimidic endonuclease (8), and recruit Rad52/Rad51 during somatic hypermutation (55). DSB formation is required for the AID-induced mutations (34). However, DSBs in the V_H locus could also occur even in the absence of AID and could occur in gene segments that did not undergo somatic mutation (4, 33). Furthermore, in the Burkitt's lymphoma cell line BL2, mutations occurred before DSBs were detected (12). Thus, whether AID induction is sufficient to account for the DSBs and mutations induced by E2-CD81 interaction remains to be investigated. It is possible that additional proteins are involved, as the DNA cleavage in V_H is dependent on de novo protein synthesis (30). Surprisingly, an error-prone DNA polymerase, polymerase ζ, is induced by E2 binding but is not involved in the E2-induced DSBs or mutations of the V_H gene. Another error-prone DNA polymerase, polymerase ι, which has previously been shown to be stimulated by HCV infection (25), is not induced by E2 binding. Thus, the functional roles of error-prone DNA poly-

merases in HCV-induced DNA mutations are very curious. It has been reported that inactivation of polymerase ζ in a Burkitt's cell line (54), or expression of *polymerase* ζ-specific antisense RNA in mice resulted in the reduction of mutation frequency (9).

Previously, we have shown that the HCV-induced nitric oxide production causes DSBs and mutations strongly in *p53*, but only slightly in the V_H gene. In the present study, we showed that the E2-CD81 interaction, conversely, enhanced mutation in V_H gene, but not in the *p53* gene. The basis for such differential effects is still not completely clear. E2-CD81 interaction may trigger a signaling response similar to that triggered by anti-CD40, interleukin-4, and other cytokines in B cells (6, 29). The normal somatic hypermutation mechanism of V_H gene in B cells typically affects the genomic sequences within ~2 kbp downstream from the transcription initiation site of Ig gene (39), under the influence of the Ig gene enhancer (16). This specificity may explain the differential effects of E2-CD81 interactions on the V_H and *p53* genes. E2 likely will bind most of cell types since CD81 is expressed ubiquitously. However, we found that E2 bound to Hep-CD81 cells but did not induce enhancement of expression of AID or DSBs in this non-B cell line. These findings suggest that the other components of the CD81 complex, including CD21 and CD19, are important for the signal transduction involved in the induction of AID (Fig. 6). Several protein kinases have been shown to associate with CD19 and CD21 but not with CD81 (13).

TNF-α is one of the earliest host responses to viral infections (19); it is an inflammatory cytokine, which can contribute directly or indirectly to viral pathogenesis. On the other hand, it may purge viruses from infected cells noncytolytically (19) and mediate intracellular signaling by adjusting the redox potential of the cell (22, 50). Since E2 of genotypes 1a and 1b have different binding affinities to CD81 and induce different amounts of TNF-α, it is conceivable that these genotypes may have different pathogenicity and may elicit different antiviral responses. Paradoxically, it has been reported that HCV genotypes 1b and 2 are associated with a high prevalence of non-Hodgkin's B-cell lymphomas (44); however, our data showed that E2 of genotype 1a induced a stronger signal to induce hypermutation and DNA damage than that of genotype 1b. Conceivably, the combined effects of DNA damage and TNF-α production may account for the relative degrees of pathogenesis of different HCV genotypes. In addition, Ig gene diversity induced by HCV may result in the divergence of specificity of antibody recognition, allowing persistent viral infection. The potential effect of E2 binding on the functions of CD81 further implicates the potential role of B cells in the pathogenesis of HCV infection.

In conclusion, engagement of B cells by E2 induces DSBs, AID, and TNF-α production. Furthermore, it leads to mutagenesis of Ig. These findings may suggest a novel therapeutic strategy based on the inhibitors of CD81 and E2.

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